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Insulin signaling in *Caenorhabditis elegans* regulates both endocrine-like and cell-autonomous outputs

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Abstract

In *C. elegans*, insulin signaling affects development, lifespan and stress resistance. Several studies have shown that insulin signaling affects lifespan in an endocrine-like manner from different cells, while the major downstream target of insulin, the FOXO transcription factor encoded by *daf-16*, may act preferentially in intestinal cells to prolong lifespan. This discrepancy raised the possibility that insulin may have both endocrine and cell-intrinsic outputs. Here, we further investigated the types of cells capable of producing endocrine outputs of insulin and also identified a new cell-intrinsic insulin output. We found that insulin signaling within groups of neurons promoted wildtype lifespan, showing that the endocrine outputs of insulin were not restricted to specific cells. In contrast, DAF-16 appeared to have a greater effect on lifespan when expressed in a combination of tissues. These results suggest that insulin signaling may regulate DAF-16 through cell-intrinsic and endocrine pathways. We also found that an insulin-dependent response to fasting in intestinal cells was preferentially regulated by intestinal insulin signaling and was less responsive to insulin signaling from non-intestinal cells. Together, these results show that *C. elegans* insulin signaling has endocrine as well as tissue-specific outputs which could influence lifespan in a combinatorial fashion.
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Introduction

In *C. elegans*, a conserved insulin-like signaling pathway promotes wildtype lifespan, stress resistance and reproductive development (Kenyon, 2005). Adult lifespan and stress resistance are increased in animals with mutations in the DAF-2/insulin receptor-like protein, AGE-1, a homolog of the p110 catalytic subunit of phosphoinositide 3-kinase (PI3K) or in AKT-1, PDK-1 or SGK-1, three downstream targets of DAF-2 (Hertweck et al., 2004; Johnson, 1990; Kenyon et al., 1993; Morris et al., 1996; Kimura et al., 1997; Paradis et al., 1999; Paradis and Ruvkun, 1998). In addition, signaling through the DAF-2 pathway is necessary to bypass arrest at the dauer larval stage, an alternative third stage larval form that is optimized for long-term survival under harsh environmental conditions (Riddle et al., 1981). The major target of insulin-like signaling is the FOXO transcription factor, DAF-16, whose mammalian orthologs are FOXO1, FOXO3a and FOXO4 (Lin et al., 1997; Ogg et al., 1997). DAF-2/IR signaling antagonizes DAF-16/FOXO via phosphorylation by AKT-1, AKT-2 and SGK-1, and promoting DAF-16's cytoplasmic retention (Henderson and Johnson, 2001; Hertweck et al., 2004; Lee et al., 2001; Lin et al., 2001; Paradis and Ruvkun, 1998). Impairment of signaling downstream of DAF-2 relieves inhibition of DAF-16 and frees DAF-16 to enter the nucleus where it may induce or repress target gene expression.

Previous studies to identify cell types where insulin signaling promotes wildtype lifespan and reproductive development in *C. elegans* showed that *daf-2* and *age-1* could function non-cell autonomously, from at least the nervous system and intestine, to promote wildtype lifespan and reproductive development (Apfeld and Kenyon, 1998; Wolkow et al., 2000). In addition, *daf-18*, a negative regulator of insulin signaling, could also promote dauer arrest and longevity from a variety of cells (Masse et al., 2005). All these studies supported the existence of endocrine outputs of insulin signaling that coordinate dauer arrest and lifespan. It was therefore unexpected to find that *daf-16* activity was apparently required in specific tissues for dauer

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arrest and longevity. Intestinal *daf-16* activity was necessary for extended lifespan in the *daf-2(e1370)* background, while *daf-16* activity in the nervous system was necessary for dauer larval arrest (Libina et al., 2003).

The discrepancies between the sites of action for *daf-16* and the upstream insulin pathway components necessitated further characterization of tissue-restricted and endocrine effects of this pathway. Here, we report that *age-1* has clear endocrine-like effects on dauer arrest and longevity. The endocrine effects of *age-1* were not restricted to specific cells, and could be produced by a variety of cells within the nervous system. We also examined the effects of cell-restricted *daf-16* activity. Although neuron- or intestine-specific *daf-16* expression had little effect on lifespan, combined expression in both tissues could lengthen lifespan of *daf-16* mutant animals. This suggests that insulin signaling may coordinate lifespan incrementally by regulating *daf-16* activity in multiple tissues, possibly through a combination of endocrine and cell-intrinsic effects. In addition, we found that insulin signaling also regulates a tissue-restricted response to fasting in intestinal cells. This suggests that not all effects of insulin signaling are mediated by endocrine outputs. Together these results support the existence of endocrine-like outputs of insulin signaling that control lifespan and dauer arrest, as well as cell-intrinsic outputs that are less responsive to endocrine regulation. These findings constitute an important step forward for understanding the complex roles of insulin in development and aging.

Materials and methods

Strains and general methods

The *C. elegans* strains used in this work were N2 Bristol (wildtype), CB1370 (*daf-2(e1370)*), GR1307 (*daf-16(mgDf50)*), WCAW196 (*age-1(mg305)*), SP75 (*sqt-1(sc13) age-1(mg44)/mnC1*); SGP296 (*sqt-1(sc13) age-1(mg109)/mnC1*); WCAW118 (*daf-16(mg242); age-1(mg109)*). The *daf-16(mg242)* mutation was isolated in a screen for suppressors of the dauer-constitutive phenotype of *age-1 (mg109)* animals and contains a nonsense mutation at tryptophan 220 that affects both the *daf-16a* and *daf-16b* splice forms. *daf-16(mg242)* completely suppresses the lifespan, stress tolerance and dauer constitutive phenotypes of *age-1(mg109)* animals. Strains were provided by the *Caenorhabditis* Genetics Center at the University of Minnesota. All strains were cultivated at 15°C on NGM agar plates seeded with the *E. coli* strain OP50 following standard protocols (Brenner, 1974).

To express a rescuing *age-1* cDNA from cell-specific promoters, a cassette strategy was used to clone each promoter as cassettes into GFP- and *age-1* cDNA-containing vectors. Promoters were amplified from *C. elegans* genomic DNA and cloned into a GFP reporter vector (pPD95.75). The promoters were also inserted into a plasmid containing the *age-1* cDNA with an *unc-54* 3′UTR, using unique restriction sites. The subcloning junctions, along with some or all of each promoter sequence, were confirmed by direct DNA sequencing. Promoters were functionally analyzed by examining the expression of GFP from the GFP reporters (Fig. S1). Primer sequences and promoter sizes are presented in Table S1.

The *gfpdaf-16* cDNA fusion was constructed by inserting *gfp* in-frame at the amino terminus of the *daf-16a* full-length cDNA yk1006c10 to create a *daf-16* construct identical to that used previously (Libina et al., 2003). Prior to subcloning, the entire *daf-16* cDNA insert in yk1006c10 was sequenced to identify any mutations. The cDNA contained one base pair that differed with the published *daf-16* sequence (t1245c), but this change is not predicted to affect the amino acid sequence of DAF-16. For insertion of *gfp*, a unique Sma*I* site was inserted at the *daf-16* amino terminus using PCR and used to insert *gfp* from pPD95.02. Promoters for *gfpdaf-16* expression were inserted upstream of *gfp* into unique Sph*I* and Kpn*I* sites. Cloning junctions and PCR-amplified sequences were confirmed by sequence analysis.

Transgenic animals were created by standard microinjection-mediated transformation of plasmid DNA at a final concentration of 25-100 μg/mL with coinjection markers expressing GFP from either the *mec-7* or *gcy-7* promoters. Transmitting lines were isolated from second generation (G2) progeny of the injected animals and the transgenic extrachromosomal arrays were crossed into the *age-1(mg44)* and *age-1(mg305)* backgrounds for phenotypic characterization. The *mg44* allele is a nonsense mutation in *age-1* that deletes the lipid kinase domain and causes a presumptive null phenotype (Morris et al., 1996). The *age-1(mg305)* allele contains a 45-nucleotide insertion in exon 3 after nucleotide 809 which results in a 15 amino acid insertion in the AGE-1 ras-binding domain, causing a reduction-of-function phenotype (Wang and Ruvkun, 2004). Transgenic animals expressing *gfpdaf-16* were constructed as for *age-1*, except that DNA was injected directly into *daf-16(mg242);age-1 (mg109)* animals. Phenotypic analyses were performed for multiple transgenic lines for each transgenic construct.

Lifespan analysis

Lifespan assays were performed at 20°C or 25°C on NGM plates supplemented with 50 μg/mL FUDR (5-fluorodeoxyuracil, Sigma) to prevent progeny overgrowth. Synchronized populations were obtained by allowing gravid adults to lay eggs at 15°C for 5-6 hours. The embryos were allowed to complete larval development at 15°C and were transferred onto FUDR-containing medium and shifted to 20°C or 25°C on the first day of adulthood, as indicated. The number of animals alive was scored every 2-3 days until death, which was defined as the failure to respond to gentle prodding on the head and tail with a platinum wire.

Statistical analysis of lifespan data was performed using the JMP software package (version 5.1). For all tables, transgenic rescue of lifespan was determined by comparing transgenic survival data against the control survival data from single experiments. Table 1 shows all survival data for

Table 1
Lifespan of control strains by experiment (for reference with Tables 2–5)

Genotype	Expt.	Mean (days)	Std error	(*n*)	% wildtype lifespan
age-1(+)	1	15.6	0.28	51	
age-1(+)	3	23.1	0.56	88	
age-1(+)	6	18.2	0.59	70	
age-1(+)	8	21.7	0.66	63	
	9	18.9	0.30	140	
	10	17.7	0.42	30	
age-1(+)	11	14.9	0.44	106	
age-1(+)	12	18.4	0.24	50	
age-1(+)	13	16.8	0.30	49	
age-1(+)	15	15.8	0.72	37	
age-1(mg44) [a]	1	22.6	1.76	22	145%
age-1(mg44) [a]	2	25.1	0.79	63	
age-1(mg44) [a]	6	24.3	1.36	39	133%
age-1(mg44) [a]	7	22.1	0.93	73	
age-1(mg44) [a]	8	26.3	0.90	92	121%
	9	22.1	0.91	46	117%
	10	23.9	0.65	45	135%
	11	19.9	0.63	98	134%
	13	19.8	1.33	26	118%
age-1(mg44) [a]	15	20.6	1.11	39	130%
age-1(mg305)	1	29.8	2.00	31	191%
	2	21.9	3.25	15	
age-1(mg305)	3	32.4	0.51	57	140%
age-1(mg305)	4	28.8	1.76	20	
	6	25.1	1.31	77	138%
age-1(mg305)	7	23.5	1.98	31	
age-1(mg305)	13	36.5	0.84	52	218%

[a] *age-1(mg44)* animals were progeny of mg44/+ hermaphrodites, were maternally-rescued for the constitutive dauer-arrest phenotype of *age-1(mg44)*.

control strains by experiment. Lifespan experiments for *age-1(mg44)* and *age-1(mg109)* controls were performed using homozygous progeny from *age-1* heterozygous hermaphrodites, which would be maternally rescued for the dauer-arrest phenotype. These animals are referred to as *m+z-* (maternal+, zygotic-) in the text. Transgenic *age-1* expression rescued dauer arrest in the *mg44* background, so it was not necessary to obtain first generation progeny from heterozygous hermaphrodites for analysis of transgenic *mg44* strains.

Dauer assays

Gravid adult hermaphrodites were allowed to lay eggs at 22°C for 4 hours and the embryos were then shifted to 25°C. Development was scored after 72 and 96 hours and the number of dauer larvae, sterile adults and fertile adults was counted.

Histochemical staining for esterase activity

Bulk esterase activity was detected in fixed animals following the protocol of Karnovsky and Roots, with slight modifications (Karnovsky and Roots, 1964). Young adult worms at the first day of egg-laying were fixed in −20°C methanol for 10 minutes and stained overnight at room temperature in a solution of 144 mM sodium acetate, 5.6 mM sodium citrate, 3.3 mM copper sulfate, 0.54 mM K3(Fe(CN)6), and 2 mM acetylthiocholine (Sigma-Aldrich, Inc., St. Louis, MO). Stained specimens were mounted on a 2% agarose pad, and viewed on a Nikon E800 microscope. Images were collected using a Hamamatsu ORCA-ER CCD camera using OpenLab software (Improvision, Lexington, MA). For fasting, animals were first cleaned of bacterial food either by washing in M9 buffer or transfer to clean NGM plates. Bacteria-free animals were transferred to NGM agar with 100 μg/mL ampicillin (to prevent bacterial growth) and incubated at 25°C for 6 hours before fixation.

Results

age-1 can act in multiple tissues to promote wildtype lifespan

Our previous study examined the ability of cell-type restricted expression of *age-1* and *daf-2* to rescue the long-lifespan phenotypes of *age-1(mg44)* and *daf-2(e1370)* mutants (Wolkow et al., 2000). In the previous study, *age-1* cDNA expression from the *unc-14* promoter rescued the Age phenotype of *age-1(mg44)* animals, while expression from the intestine-specific *ges-1* or muscle-specific *unc-54* promoters did not. However, we wished to further investigate a requirement for *age-1* expression in the intestine and muscles. To do this, we integrated the *Pges-1:age-1* and *Punc-54:age-1* transgenes by UV-irradiation, backcrossed them 4 times into the wildtype background and then crossed them into the *age-1* mutants. The integrated transgenes were able to rescue long lifespan of both *age-1(mg305)* and *age-1(mg44)*

Table 2
Adult lifespan of animals with *age-1* expression restricted to neurons, intestine or muscle

Genotype	Mean (days)	Std error	(*n*)	Expt.	% shortened	*P* vs control[a]
Pan-neuronal (Pric-19:age-1)						
age-1(+); bvIs2	13.3	0.48	73	13	20	<0.0001, <0.0001
age-1(+); bvIs2	14.4	0.65	56	13	14	0.2209, 0.0603
age-1(mg44); bvIs2	19.9	0.77	74	7	10	0.0010, 0.0319
age-1(mg44); bvIs2	24.3	1.37	45	8	8	0.1131, 0.2136
age-1(mg305); bvIs2	19.7	0.77	122	7	16	0.0003, 0.0961
age-1(mg305); bvIs2	21.3	1.02	44	13	58	<0.0001, <0.0001
age-1(+); bvEx125	15.5	0.62	42	13	7	0.5718, 0.0699
age-1(+); bvEx128	16.6	0.78	49	13	1	0.0335, 0.5328
age-1(mg44); bvEx11	17.8	0.88	45	1	21	0.0004, 0.0192
age-1(mg44); bvEx12	14.3	0.29	78	1	37	<0.0001, <0.0001
age-1(mg44); bvEx12	16.7	0.64	81	11	16	<0.0001, <0.0001
age-1(mg44); bvEx12	16.7	0.64	81	11	16	<0.0001, <0.0001
age-1(mg44); bvEx120	21.1	0.67	119	9	5	0.8562, 0.5176
age-1(mg44); bvEx121	21.9	0.43	85	9	1	0.0133, 0.1121
age-1(mg44); bvEx122	20.2	0.45	135	9	9	0.0001, 0.0028
age-1(mg44); bvEx123	19.3	0.56	92	9	13	<0.0001, 0.0004
age-1(mg44); bvEx123	19.6	0.69	41	10	18	<0.0001, <0.0001
age-1(mg44); bvEx125	19.3	0.88	50	9	13	0.0091, 0.0095
age-1(mg44); bvEx125	17.4	0.90	49	10	27	<0.0001, <0.0001
age-1(mg44); bvEx125	19.0	0.54	61	13	4	0.0248, 0.1169
age-1(mg44); bvEx128	22.0	1.01	26	9	0	0.5992, 0.4764
age-1(mg44); bvEx128	16.6	0.78	49	13	16	0.0002, 0.0102
age-1(mg44); bvEx128	18.1	0.56	36	15	12	<0.0001, 0.0030
age-1(mg305);bvEx(ric-19:age-1)	26.0	1.14	79	3	20	<0.0001,<0.0001
Intestine (Pges-1:age-1)						
age-1(mg44); bvIs1	20.5	0.72	50	8	22	<0.0001, <0.0001
age-1(mg305); bvIs1	21.3	1.05	47	1	6	<0.0001, <0.0001
age-1(mg305); bvIs1	27.0	1.06	77	3	17	<0.0001,<0.0001
Body muscle (Punc-54:age-1)						
age-1(mg44); mgIs37	21.4	1.41	44	8	19	0.0057, 0.0067
age-1(mg305); mgIs37	19.7	0.85	45	1	34	<0.0001, <0.0001
age-1(mg305); mgIs37	23.6	0.99	75	3	27	<0.0001,<0.0001

[a] Log-Rank, Wilcoxan, versus non-transgenic control.

strains (Table 2). These positive results negate the previously published negative results for *age-1*, and they are consistent with the previous finding that intestine-restricted *daf-2* expression shortened *daf-2(e1370)* lifespan.

We also re-examined whether neural-specific *age-1* activity rescued lifespan in *age-1(-)* strains by driving *age-1* expression from the neural-specific promoter *Pric-19* (Pilon et al., 2000). Lifespan was rescued in animals carrying *Pric-19:age-1* transgenes (Fig. 1A, Table 2). In most experiments, *age-1(-)* animals with extrachromosomal *Pric-19:age-1* transgenes lived 12-37% shorter than nontransgenic controls, while these transgenes only shortened wildtype lifespan by 1-7%. An integrated *Pric-19:age-1* transgene also shortened lifespan of *age-1(mg305)* animals (16-58% shorter), while reducing wildtype lifespan approximately 20% (Fig. 1A, *bvIs2*). The negative effect of the integrated array in the wildtype strain could be due to the presence of second site mutations possibly incurred during UV-irradiation. Since transgenic *age-1* expression had more significant effects on lifespan of *age-1(-)* animals than wildtype animals, we conclude that the transgenes specifically rescued the long lifespan phenotype of *age-1(-)* rather than nonspecifically shortening lifespan.

To further verify that intestinal *age-1* expression could rescue long lifespan of *age-1(-)* animals, we constructed animals expressing the *age-1* cDNA from the promoters for the intestinal genes, *gly-19* and *spl-1* (Mendel et al., 2003; Warren et al., 2001). Both the *gly-19* and *spl-1* promoters directed robust expression of GFP reporters through late life (not shown). Expression of *age-1* from *gly-19* and *spl-1* promoters also robustly rescued long lifespan in both the *age-1(mg44)* and *age-1(mg305)* backgrounds (Fig. 1B, Table 3). In *age-1(mg44)* animals carrying *age-1* expressed from either the *gly-19* or *spl-1* promoters, lifespan was reduced between 16-46% (Table 2). The same transgenes only slightly reduced lifespan of *age-1(+)* animals (0-14% reduction). These results confirm the findings with the integrated *Pges-1:age-1* array.

We further analyzed this survival data to determine whether insulin signaling regulates lifespan in a binary "all-or-none" fashion or promoted fractional changes in lifespan. If insulin were to act in a binary fashion, then transgenic populations should consist of a mixture of rescued and non-rescued subpopulations, resulting in survival curves with features of both subpopulations. Such a pattern has been observed in heterozygous populations of animals with single-gene mutations that affect lifespan (Friedman and Johnson, 1988). Similar binary effects on lifespan have also been observed in genetic mutants that promote long lifespan only in a fraction of the total population, without altering lifespan of all animals (Gerisch and Antebi, 2004; Nanji et al., 2005). Alternatively, if insulin regulates lifespan through fractional effects, then survival data

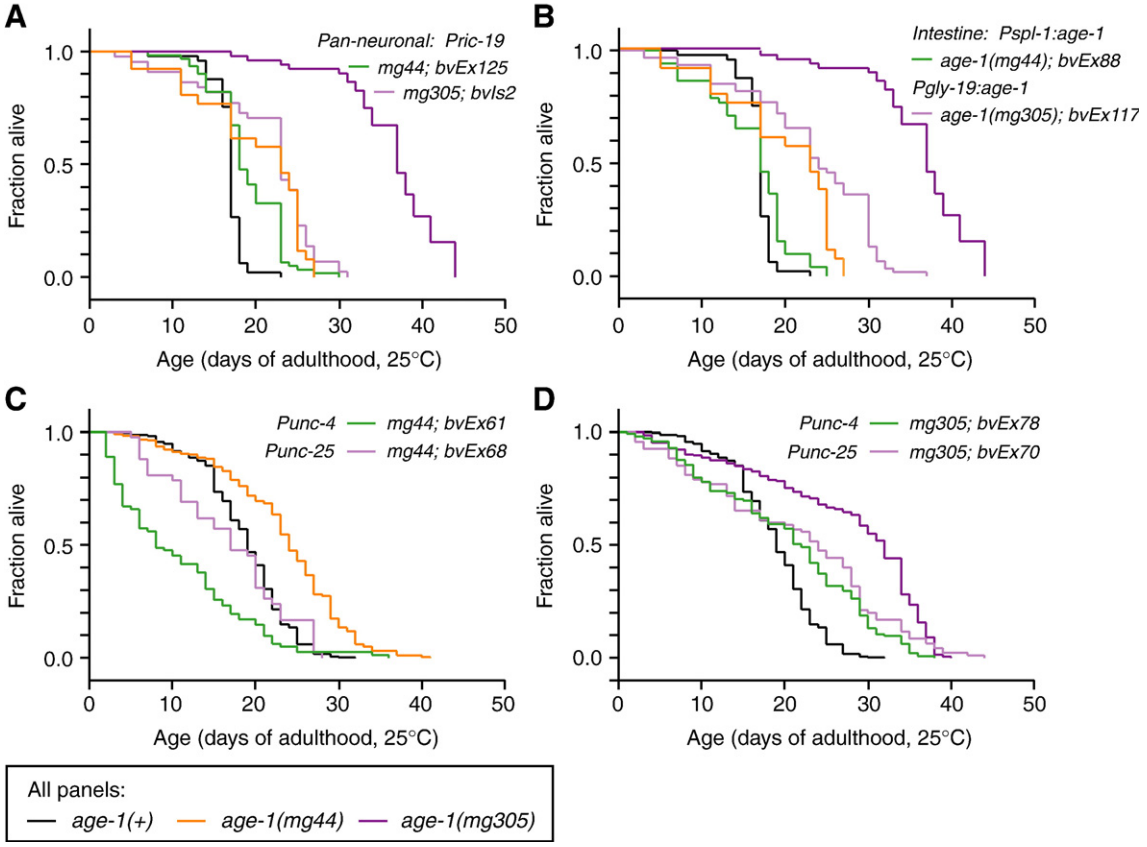

Fig. 1. Rescue of long lifespan by neuron- or intestine-specific *age-1* expression. (A, B) Results from one representative experiment; (C, D) cumulative survivorship. Data for individual trails presented in Tables 1–4. In all panels, black designates wildtype *age-1(+)*, orange designates *age-1(mg44)* adults maternally rescued for dauer arrest, dark purple designates *age-1(mg305)* adults. Transgenic lines as follows: (A) Pan-neuronal *age-1* (*Pric-19:age-1*); (B) intestine-specific *age-1* (*Pspl-1:age-1* or *Pgly-19:age-1)*; (C, D) green, cholinergic motor neuron-specific *age-1* (*Punc-4:age-1*); light purple, GABAergic motorneuron-specific *age-1* (*Punc-25:age-1*).

Table 3
Adult lifespan of animals with intestine-restricted *age-1*

Genotype	Mean (days)	Std error	(*n*)	Expt.	% Shortened	*P* vs control[a]
age-1(+); bvEx86	16.3	0.54	48	13	3	0.3217, 0.6986
age-1(+); bvEx87	14.4	0.79	26	13	14	0.2814, 0.0504
age-1(+); bvEx87	18.2	0.66	38	15	+15	0.0072, 0.0136
age-1(+); bvEx88	16.3	0.30	56	13	3	0.5703, 0.4297
age-1(+); bvEx89	15.9	0.36	67	13	5	0.7575, 0.3576
age-1(+); bvEx90	16.7	0.42	30	13	0	0.3238, 0.3575
age-1(+); bvEx90	19.4	0.53	33	15	22	0.0006, 0.0011
age-1(+); bvEx117	16.7	0.76	44	13	1	0.0053, 0.0662
age-1(mg44); bvEx86	17.9	1.21	44	8	32	<0.0001, <0.0001
age-1(mg44); bvEx87	11.9	1.23	24	7	46	<0.0001, <0.0001
age-1(mg44); bvEx87	21.3	1.94	29	8	19	0.1057, 0.0160
age-1(mg44); bvEx87	17.4	0.66	64	9	21	<0.0001, <0.0001
age-1(mg44); bvEx87	25.2	1.36	19	15	+22	0.0366, 0.0868
age-1(mg44); bvEx88	13.2	1.18	38	7	40	<0.0001, <0.0001
age-1(mg44); bvEx88	15.9	0.71	52	13	20	0.0001, 0.0056
age-1(mg44); bvEx89	17.8	1.81	31	8	32	0.0009, <0.0001
age-1(mg44); bvEx89	16.1	1.16	16	15	22	0.0011, 0.0051
age-1(mg44); bvEx90	12.8	1.04	51	7	42	<0.0001, <0.0001
age-1(mg44); bvEx90	16.6	0.93	60	13	16	0.0166, 0.0438
age-1(mg305); bvEx117	23.1	1.03	61	13	63	<0.0001, <0.0001

[a] Log-Rank, Wilcoxan, versus non-transgenic control.

for partially rescued populations should show features that are intermediate between the wildtype and non-transgenic mutant controls. Such fractional effects on lifespan are observed for calorie restriction and temperature in *C. elegans*, where a range of dietary and environmental conditions causes incremental changes in lifespan (Houthoofd et al., 2003; Johnson et al., 1984; Klass, 1977).

To address this question, we created exponential plots of the survival data for *Pric-19:age-1* and *Pgly-19:age-1* or *Pspl-1:age-1* transgenic animals, and compared these curves to wildtype and non-transgenic controls. These transgenes appeared to promote incremental rescue of *age-1(-)* lifespan. In both the case of neuron- and intestine-restricted *age-1* expression, mortality increased at ages that were intermediate between wildtype and non-transgenic controls (Fig. 2). This incremental behavior was most pronounced for transgenes in the *age-1(mg305)* background, which had a stronger effect on lifespan. Transgenic rescue of lifespan in *age-1(mg44)* animals was closer to that of wildtype. This analysis suggests that endocrine-like outputs of insulin signaling affect lifespan in a graded fashion from several different tissues.

age-1 can act in several types of neurons to promote wildtype lifespan and reproductive development

To determine whether specific neurons could regulate lifespan, or if insulin signaling were required throughout the nervous system, we used a similar transgenic approach to

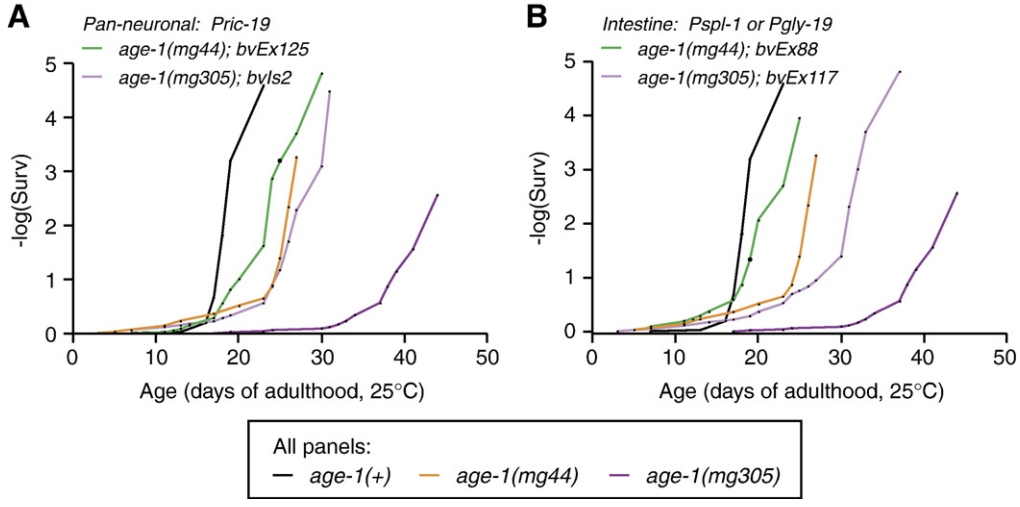

Fig. 2. Exponential plot of survival data shown in Figs. 1A and B. Neuron- or intestine-restricted *age-1* expression was correlated with mortality increases that were intermediate between wildtype and non-transgenic control animals.

express *age-1* in groups of neurons. The rescuing *age-1* cDNA was restricted to subsets of neurons as follows: 32 motor neurons using the *unc-4* promoter, 26 inhibitory motor neurons using the *unc-25* promoter, or to 8-11 interneurons using the *flp-1* promoter (McIntire et al., 1993; Miller et al., 1992; Nelson et al., 1998; White et al., 1992). Restricted *age-1* expression to these subsets of neurons partially rescued lifespan *age-1(-)* animals (Figs. 1C, D, Table 4). Interneuron-restricted *age-1* expression shortened lifespan in *age-1(-)* animals by 25-45%, but had a negligible effect on lifespan of *age-1(+)* animals (0-7% shorter). Motor-neuron-restricted *age-1* expression also significantly shortened lifespan of *age-1(mg44)* animals (*Punc-4:age-1*, 39-60% shorter than controls; *Punc-25:age-1*, 18-56% shorter). Motor-neuron-restricted *age-1* expression had weaker effects on *age-1(mg305)* lifespan (*Punc-4:age-1*, 11-18% shorter; *Punc-25:age-1*, 0-31% shorter). In control experiments with wildtype animals, the *Punc-4:age-1* transgene shortened lifespan by 5-14%, although the *Punc-25:age-1* transgene had negligible effects (0-4% shorter). These results suggest that insulin signaling in groups of neurons can contribute, at least partially, to normal lifespan. An exponential plot of the survival data confirmed that mortality increased at ages intermediate between wildtype and non-transgenic controls (not shown). This supports the hypothesis that insulin signaling in different groups of neurons can affect lifespan in a graded fashion.

In addition to rescuing lifespan, each promoter tested provided sufficient *age-1* activity to rescue the dauer arrest phenotype of *age-1(mg44)* and *age-1(mg305)* animals (Fig. 3, Table 5). These findings are consistent with earlier studies showing that *daf-2*, *age-1* and *daf-16* promoted reproductive development in an endocrine-like manner primarily from neurons (Apfeld and Kenyon, 1998; Libina et al., 2003; Wolkow et al., 2000). However, the level of rescue varied between transgenic lines. In some lines, such as *bvEx122*, reproductive development was fully restored. Full rescue was usually correlated with *age-1* expression from the pan-neuronal *ric-19* promoter. In other cases, such as *bvEx47*, dauer arrest was partially rescued and a majority of the transgenic larvae

Table 4
Lifespan of animals with transgenic *age-1* in groups of neurons

Genotype	Mean (days)	Std error	(*n*)	Expt.	% Shortened	*P* vs control[a]
Interneurons						
Pflp-1:age-1						
age-1(+); bvEx141	18.7	0.30	46	12	+2	0.5305, 0.7701
age-1(+); bvEx142	17.0	0.34	47	12	7	0.0028, 0.0017
age-1(+); bvEx140	18.0	0.32	47	12	2	0.4283, 0.3017
age-1(mg44); bvEx47	14.4	2.34	13	8	45	<0.0001, <0.0001
age-1(mg305); bvEx47	23.1	0.82	105	4	20	0.0006, 0.0050
Motor neurons						
Punc-4:age-1						
age-1(+); bvEx131	17.1	0.45	36	12	7	0.0243, 0.0106
age-1(+); bvEx132	16.6	0.49	49	12	10	0.0077, 0.0017
age-1(+); bvEx133	15.9	0.28	28	12	14	<0.0001, <0.0001
age-1(+); bvEx134	16.0	0.64	25	12	13	<0.0001, <0.0001
age-1(+); bvEx135	16.7	0.43	53	12	9	0.0084, 0.0026
age-1(+); bvEx136	17.4	0.32	24	12	5	0.0242, 0.0246
age-1(mg44); bvEx61	12.7	1.48	29	2	49	<0.0001, <0.0001
age-1(mg44); bvEx61	9.6	1.06	53	6	60	<0.0001, <0.0001
age-1(mg44); bvEx62	15.2	0.86	44	2	39	<0.0001, <0.0001
age-1(mg305); bvEx62	19.6	1.20	77	2	11	0.1209, 0.4104
age-1(mg305); bvEx75	21.8	1.11	116	6	13	0.1071, 0.0474
age-1(mg305); bvEx78	20.5	0.95	102	6	18	<0.0001, 0.0021
Punc-25:age-1						
age-1(+); bvEx137	18.2	0.25	58	12	1	0.5807, 0.4141
age-1(+); bvEx138	17.7	0.28	40	12	4	0.0659, 0.0537
age-1(+); bvEx139	18.4	0.32	42	12	0	0.9592, 0.7579
age-1(mg44); bvEx67	20.6	1.33	15	2	18	0.0010, 0.0010
age-1(mg44); bvEx68	16.7	1.11	42	2	33	<0.0001, <0.0001
age-1(mg44); bvEx69	12.7	0.87	56	2	49	<0.0001, <0.0001
age-1(mg44); bvEx70	11.1	0.85	34	2	56	<0.0001, <0.0001
age-1(mg305); bvEx67	15.1	0.99	24	2	31	0.0064, 0.1304
age-1(mg305); bvEx67	24.0	1.35	70	6	4	0.1681, 0.3491
age-1(mg305); bvEx68	21.3	2.36	12	2	3	0.8320, 0.9221
age-1(mg305); bvEx68	22.8	1.07	112	6	9	0.1136, 0.1237
age-1(mg305); bvEx69	23.7	1.72	27	2	+8	0.7893, 0.9043
age-1(mg305); bvEx69	21.4	1.39	94	6	15	0.8705, 0.0665
age-1(mg305); bvEx70	21.6	1.14	95	6	14	0.0364, 0.0225

[a] Log-Rank, Wilcoxan, versus non-transgenic control.

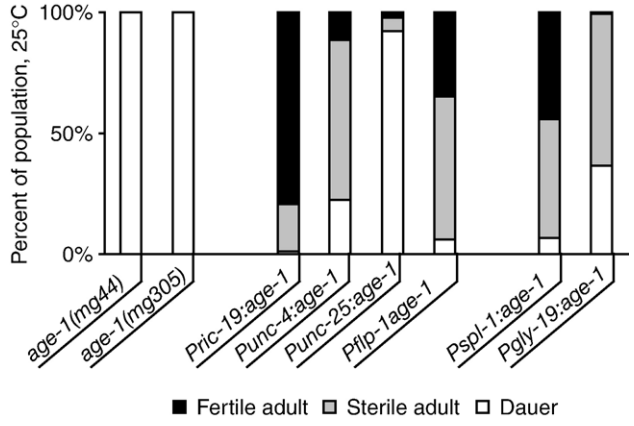

Fig. 3. Rescue of dauer larval arrest by neuron- or intestine-specific *age-1* expression. Rescue of dauer larval arrest was assayed at 25°C as the fraction of animals arrested as dauer larvae (white), or who had completed development to fertile (black) or sterile (grey) adults after 72 hours. Fertile and sterile adults were differentiated by the presence or absence of eggs in the uterus in fertile adults. Graph shows cumulative data for all independent lines from each transgene; 1-5 lines were tested for each transgene. Results for individual lines are presented in Table 5.

developed into sterile adults. Weak or partial rescue of dauer arrest was observed primarily when *age-1* was expressed from subsets of neurons or in the intestine. Partial rescue of dauer arrest by *age-1* expression in groups of neurons is also consistent with a graded effect of insulin signaling upon development, as observed for lifespan.

daf-16 may act in combinations of tissues to incrementally increase lifespan

The FOXO transcription factor, DAF-16, is the ultimate output of signaling by DAF-2 and AGE-1 and is required for extended lifespan in animals with defective DAF-2 pathway signaling (Ogg et al., 1997). Previous analysis showed that *daf-16* activity in the intestine was necessary to prolong lifespan, while neuron-restricted *daf-16* activity promoted dauer arrest but had no effect on lifespan (Libina et al., 2003). Given the endocrine-like effect of *age-1* on lifespan, we asked whether *daf-16* function in multiple tissues could have additive effects on lifespan. If this were the case, then lifespan could reflect the regulation of *daf-16* through a combination of endocrine and tissue-restricted outputs of insulin.

We constructed a GFP-tagged *daf-16* cDNA construct identical to that used previously, which was expressed either from the native *daf-16a* promoter, the ubiquitous *dpy-30* promoter, the neuronal *ric-19* promoter or the intestinal *gly-19* or *spl-1* promoters. We then examined whether transgenic *daf-16* expression in these tissues could rescue the dauer-defective and shortened lifespan phenotypes in a *daf-16(mg242); age-1 (mg109)* double mutant background. Expression of *gfp:daf-16* from either the ubiquitous *dpy-30* promoter or the endogenous *daf-16a* promoter weakly rescued dauer arrest in *daf-16 (mg242);age-1(mg109)* animals, although these transgenes were associated with high rates of embryonic lethality. In particular, 87% of transgenic progeny died as embryos, 6% arrested as dauer or dauer-like larvae and the remaining 7% grew to adulthood, among the first generation progeny of hermaphrodites injected with the *Pdpy-30:gfp:daf-16* construct (not shown). Due to the high level of embryonic lethality, we were only able to isolate one line which could transmit the *Pdpy-30:gfp:daf-16* transgene. Dauer arrest was not observed in this line, but lifespan was lengthened by 25% (not shown, Fig. 4A, Table 6).

Expression of *gfpdaf-16* from either the neuronal *ric-19* or the intestinal *gly-19* or *spl-1* promoters in *daf-16(mg242); age-1(mg109)* animals did not cause embryonic lethality or dauer arrest (not shown). In addition, neuronal or intestinal

Table 5
Developmental phenotype of *age-1(-)* animals with cell-type restricted *age-1* expression

Promoter	Parental genotype	Developmental phenotype (25°C)				Rescue development (Y/P/N) *
		Dauers (%)	Adults (%)		*n*	
			Fertile	Sterile		
	age-1(mg44) m+z-	100.0	0.0	0.0	133	
	age-1(mg305)	100.0	0.0	0.0	183	
ric-19	*mg44; bvEx120*	1.3	79.6	19.1	152	Y
	mg44; bvEx122	0.0	93.4	6.6	198	Y
	mg44; bvEx123	1.9	87.5	10.6	104	Y
	mg44; bvEx125	0.9	50.5	48.6	111	Y/P
	mg44; bvEx128	0.9	86.0	13.1	107	Y
unc-4	*mg305; bvEx78*	22.4	11.4	66.2	210	P
unc-25	*mg305; bvEx68*	92.2	2.2	5.6	90	N/P
flp-1	*mg44; bvEx47*	10.1	9.6	80.3	208	P
	mg305; bvEx47	2.0	60.1	37.9	198	Y
spl-1	*mg44;bvEx87*	0.0	28.6	71.4	28	P
	mg305;bvEx88	25.9	0.9	73.2	112	P
	mg44; bvEx90	0.0	75.0	25.0	52	Y
	mg305;bvEx90	1.3	72.7	26.0	231	Y
gly-19	*mg305;bvEx117*	36.6	0.7	62.7	279	P

∗ Rescue: Yes/Partial/None.

daf-16 expression had weak effects on lifespan (*Pric-19:gfpdaf-16*: 8-18% increased lifespan; *Pgly-19:gfpdaf-16* 0-14% increased lifespan, Fig. 4A, Table 6). We also examined the effect of expressing *daf-16* in both neurons and intestine together. Co-transformation of *daf-16(mg242); age-1(mg109)* animals with both the *Pric-19:gfp:daf-16* and *Pgly-19:gfp:daf-16* transgenes also did not affect dauer arrest, but did lengthen lifespan more than *daf-16* expression in either tissue alone (Fig. 4A, Table 6). An exponential plot of the survival data for transgenic *daf-16* expression suggests an incremental effect of increased *daf-16* expression on lifespan, with the least lifespan-lengthening activity in animals with only neuronal *daf-16*, slightly more in animals with neuron + intestine *daf-16* and the greatest in animals with ubiquitous *daf-16* activity (Fig. 4B).

Insulin signaling affects a cellular response to fasting

The transgenic studies suggested that insulin signaling has graded effects on development and lifespan, possibly through endocrine-like outputs. We therefore wished to investigate whether insulin also had cell-intrinsic outputs. To address this question, we developed an assay for monitoring fasting response at the cellular level in the intact organism. Esterase enzymes are highly abundant in the *C. elegans* intestine where they function to assist digestion and detoxification of injested material. *In situ* staining for esterase activity in wild type young adult animals revealed cytoplasmic distribution of esterase activity in the intestines of well-fed animals (Fig. 5A). We found that fasting caused a dramatic alteration in the subcellular distribution of esterase activity. When wild-type young adult animals were kept without food for six hours, intestinal esterase activity redistributed from the cytoplasm to the nucleus (Fig. 5B). We refer to this as the FIRE response (Fasting-Induced Redistribution of Esterase activity). Insulin signaling was required for a normal FIRE response to fasting. When *daf-2(e1370)* or *age-1(mg305)* animals were fasted for 6 hours, intestinal esterase activity remained cytoplasmic. (Figs. 5C, D). The altered FIRE response in insulin pathway mutants required *daf-16* activity, as *daf-16 (mgDf50);age-1(mg305)* animals displayed the wildtype FIRE response to fasting (Fig. 5E). The basis for the defective FIRE response to fasting in insulin pathway mutant strains may be due to altered metabolism in these animals, which have been shown to accumulate high levels of fat and may be hypometabolic (Halaschek-Wiener et al., 2005; Holt and Riddle, 2003; Kimura et al., 1997).

We next asked whether the endocrine-like outputs of insulin regulated the FIRE response by examining this response in the transgenic strains with tissue-restricted *age-1* and *daf-16* expression. In most cases, neuron-restricted *age-1* expression did not rescue the altered FIRE response. In particular, *age-1* expression from the pan-neuronal *ric-19* or the motorneuron-specific *unc-25* promoters failed to rescue the altered FIRE response of *age-1(-)* animals, although these transgenes could rescue lifespan and dauer arrest (Figs. 5F, 6, Table 7). Intestinal *age-1* expression from the *spl-1* promoter restored the normal FIRE response in about 40% of transgenic animals, indicating partial rescue of this phenotype (Fig. 5G, Table 7). Consistent results were obtained for *daf-16*. Ubiquitous or intestinal *daf-16* expression had the strongest effect on promoting an altered FIRE response in *daf-16(mg242); age-1(mg109)* animals (Table 7). Neuron-restricted *daf-16* expression had a much weaker effect on this response. The observation that the FIRE response was better restored by intestinal insulin signaling than by neuronal insulin signaling suggests that this response reflects an intestine-intrinsic insulin output.

Discussion

Insulin signaling in *C. elegans* controls dauer developmental arrest, lifespan and stress resistance. Here, we extended previous

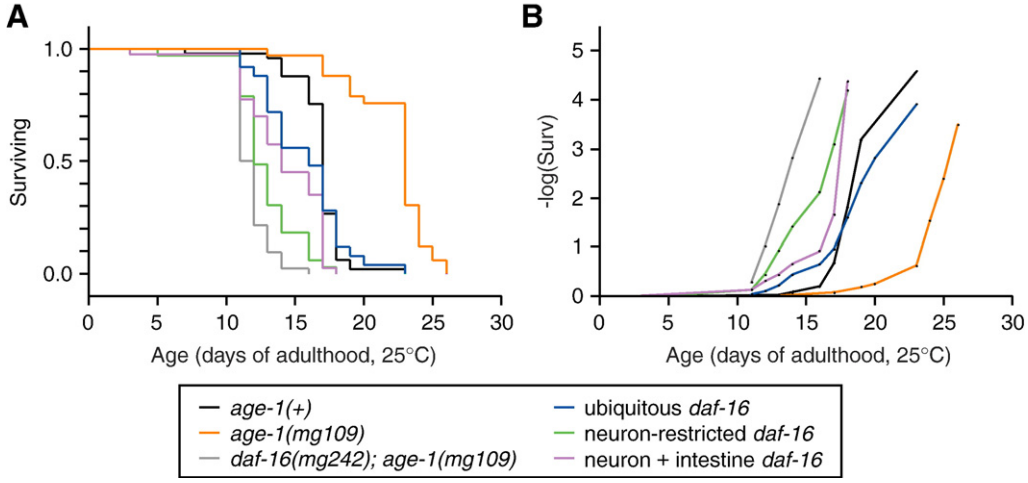

Fig. 4. Lifespan in animals with tissue-restricted *daf-16* expression. (A) Survival plot or (B) exponential plot of survival data for wildtype (black) or *daf-16(mg242); age-1(mg109)* (grey) fertile adults at 25°C. Transgenic *daf-16* activity was provided by expressing *gfpdaf-16* in the *daf-16(mg242); age-1(mg109)* background, as follows: ubiquitous *gfpdaf-16* (*Pdpy-30:gfpdaf-16*, blue), neuron-specific *gfpdaf-16* (*Pric-19:gfpdaf-16*, green), or *gfpdaf-16* in both neurons and intestine (*Pgly-19:gfpdaf-16* + *Pric-19:gfpdaf-16*, purple). Results from one representative trial are presented. Complete lifespan statistics are presented in Table 6.

Table 6
Adult lifespan of animals with tissue-restricted transgenic expression of *gfp:daf-16*

Genotype	Mean (days)	Std error	(*n*)	Expt.	% Increase	*P* vs control[a]
daf-16(+); age-1(+)	15.3	0.74	38	1	31	
daf-16(+); age-1(+)	19.2	0.48	85	2	41	
daf-16(+); age-1(+)	16.8	0.30	49	3	29	
daf-16(+); age-1(+)	19.7	1.12	36	4[b]	25	
age-1(mg109) m+z-	22.2	0.51	33	3	46	
age-1(mg109) m+z-	35.5	1.86	23	4[b]	59	
daf-16(mg242); age-1(mg109)	10.6	0.31	42	1		
daf-16(mg242); age-1(mg109)	11.3	0.67	21	2		
daf-16(mg242); age-1(mg109)	11.9	0.18	42	3		
daf-16(mg242); age-1(mg109)	14.7	0.19	61	4[b]		
Ubiquitous daf-16 (Pdpy-30:daf-16)						
daf-16(-); age-1(-); bvEx130	15.7	0.59	25	3	25	<0.0001, <0.0001
daf-16(-); age-1(-); bvEx130	19.5	0.90	29	4[b]	25	<0.0001, <0.0001
Neuronal daf-16 (Pric-19:daf-16)						
daf-16(-);age-1(-); bvEx94	13.0	0.41	46	1	18	<0.0001, <0.0001
daf-16(-);age-1(-); bvEx94	12.8	0.41	33	3	7	0.0023, 0.0037
daf-16(-);age-1(-); bvEx94	16.0	0.71	28	4[b]	8	0.0043, 0.0883
daf-16(-);age-1(-); bvEx92	11.1	0.32	35	1	5	0.2902, 0.2660
daf-16(-);age-1(-); bvEx93	12.8	0.36	46	1	17	<0.0001, <0.0001
daf-16(-);age-1(-); bvEx96	11.3	0.48	40	1	6	0.0578, 0.2336
daf-16(-);age-1(-); bvEx98	11.8	0.50	24	1	10	0.0744, 0.0499
Intestinal daf-16 (Pgly-19:daf-16, Pspl-1:daf-16)						
daf-16(+); bvEx103	18.5	0.36	76	2	−4	0.0160, 0.0256
daf-16(+); bvEx105	19.3	0.43	79	2	0	0.6309, 0.5742
daf-16(-); age-1(-); bvEx#13	11.9	0.88	7	1	11	0.1244, 0.2050
daf-16(-); age-1(-); bvEx#P1	12.3	0.65	19	1	14	0.0278, 0.0300
Neuronal + intestinal daf-16 (Pric-19:daf-16 + Pgly-19:daf-16)						
daf-16(-); age-1(-); bvEx113	14.5	0.45	62	2	22	<0.0001, <0.0001
daf-16(-); age-1(-); bvEx113	14.6	0.46	39	3	18	<0.0001, <0.0001
daf-16(-); age-1(-); bvEx113	18.0	0.61	53	4[b]	18	<0.0001, <0.0001
daf-16(-); age-1(-); bvEx114	14.9	0.61	31	2	24	<0.0001, <0.0001
daf-16(-); age-1(-); bvEx114	14.1	0.48	40	3	16	<0.0001, <0.0001
daf-16(-); age-1(-); bvEx114	19.3	0.98	28	4[b]	24	<0.0001, <0.0001

[a] Log-Rank, Wilcoxan, versus non-transgenic control.
[b] Experiment 4 was performed at 20°C; Expts. 1-3 were performed at 25°C.

observations by showing that insulin signaling in groups of neurons could promote wildtype lifespan and rescue dauer arrest. These findings suggest equivalency throughout the nervous system for endocrine-like outputs of insulin that regulate these phenotypes. Animals with insulin signaling restored to small groups of cells appeared to have generally weaker rescue of these phenotypes than when insulin signaling were restored to larger groups of cells. A similar result was observed from analysis of *daf-2* mosaic animals (Apfeld and Kenyon, 1998). This may reflect a quorum or graded effect of insulin in which insulin signaling within a threshhold number of cells is necessary for reproductive development and wildtype lifespan.

We also examined the effect of insulin on a response to fasting, the FIRE response, within individual intestinal cells. To our knowledge, this is the first study to examine the effects of *C. elegans* insulin signaling at a cellular level. These studies demonstrated that the lifespan and developmental phenotypes of *age-1* mutants could be separated from the altered FIRE response within intestinal cells. These results suggest a model of insulin signaling where endocrine-like outputs of insulin coordinate lifespan and dauer developmental arrest, while cell-intrinsic outputs of insulin affect cellular responses, such as the FIRE response (Fig. 7).

DAF-16/FOXO is the major effector of insulin signaling in *C. elegans* and is required for dauer formation, stress resistance and long lifespan in insulin-defective mutants (Kenyon et al., 1993; Lin et al., 1997; Ogg et al., 1997). Evidence that AKT directly phosphorylates FOXO proteins suggests that DAF-16 must function in the same cells as AKT-1, and, by extension, AGE-1 and DAF-2 (Brunet et al., 1999; Paradis and Ruvkun, 1998). Previous analysis of *daf-16* sites of function revealed that intestine-specific *daf-16* expression could increase lifespan of *daf-16(mu86); daf-2 (e1370)* animals (Libina et al., 2003). In contrast, neuron-specific *daf-16* expression did not affect lifespan, but did promote dauer arrest. Additive effects of *daf-16* activity in these tissues were not investigated. In the present study, we

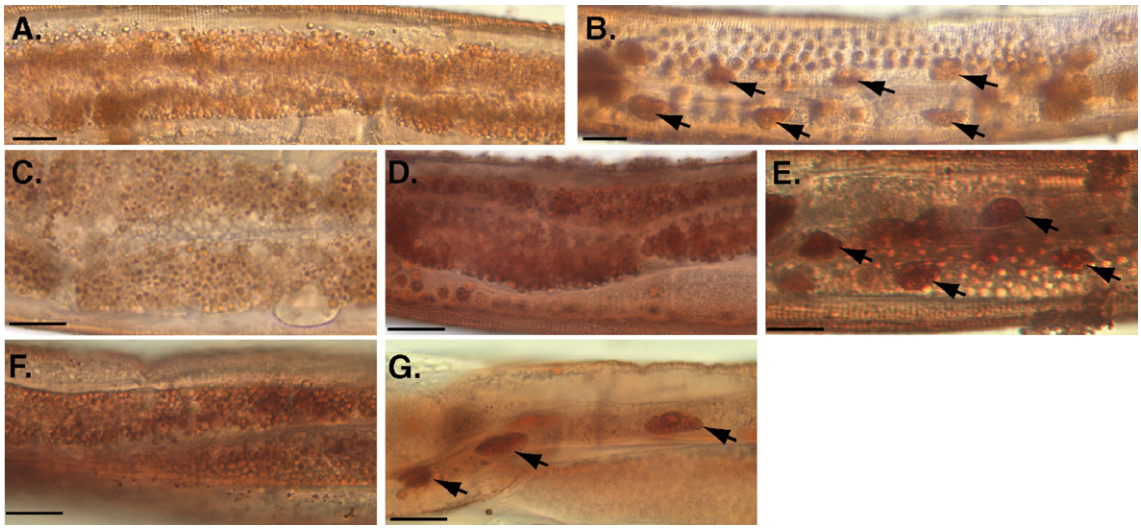

Fig. 5. Altered FIRE response to fasting in insulin pathway mutants. (A) *In situ* detection of cytoplasmic esterase activity in intestinal cells of well-fed young adult animals. (B) Intestinal esterase activity in a young adult after 6-hour fast displayed FIRE response by redistribution of esterase activity to intestinal nuclei (arrows). (C-E), Insulin signaling modulates the FIRE response to fasting. Intestinal esterase activity remained cytoplasmic in (C) *daf-2(e1370)* or (D) *age-1(mg305)* young adult animals after 6-hour fast. (E) *daf-16* was required for fasting resistance of *daf-2* pathway mutants; the wildtype nuclear pattern for esterase localization was restored in *daf-16(mgDf50); age-1(mg305)* young adults after fasting. (F) Pan-neuronal *age-1* expression did not rescue altered FIRE response of *age-1(mg44)* animals (age-*1 (mg44); bvEx123*); (G) Intestine-specific *age-1* expression rescued FIRE response, (age-*1(mg44); bvEx87*). Scale bars, 20 μm. Complete data for FIRE response phenotype is presented in Table 7.

found that intestine-specific or neuron-specific *daf-16* expression did not restore long lifespan to *daf-16(mg242); age-1 (mg109)* animals, although *daf-16* expression in both tissues did increase lifespan, suggesting that lifespan may also be affected in an incremental fashion by DAF-16 activity in multiple tissues.

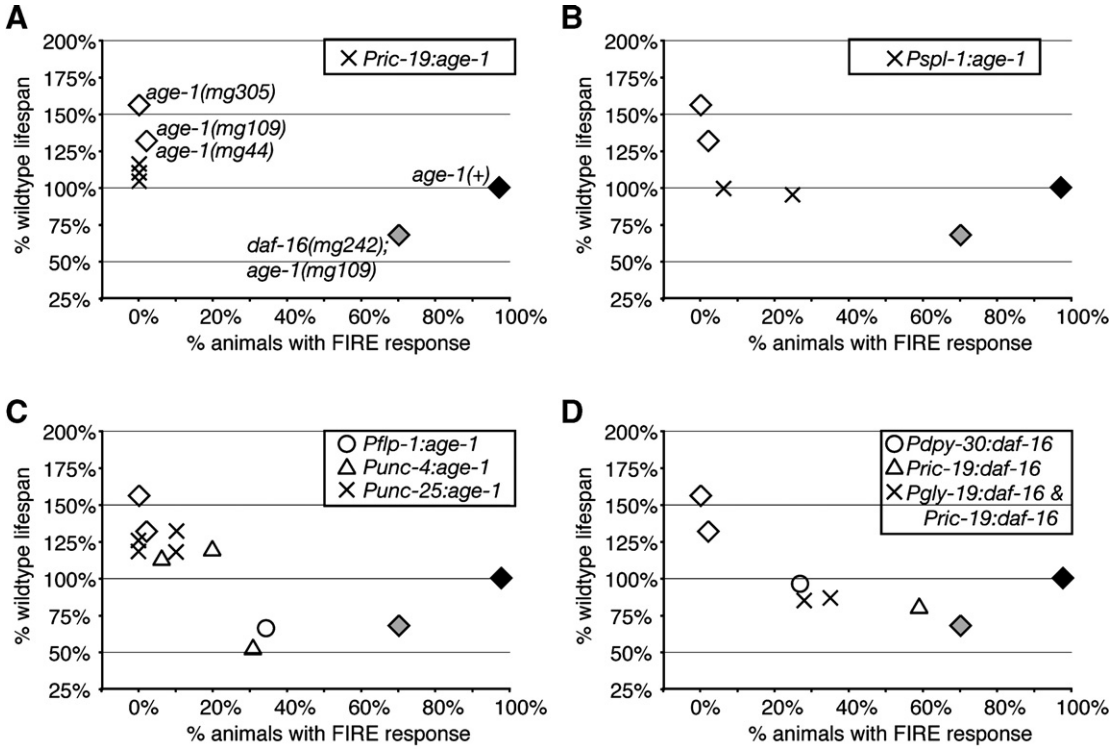

Fig. 6. Effects of tissue-specific *age-1* expression on lifespan and FIRE response. Adult lifespan and FIRE response were plotted to examine the correlation between these phenotypes. In all panels, black diamond, wildtype; white diamonds are *age-1(lf)* alleles, as indicated in (A); grey diamond, *daf-16(mg242); age-1(mg109)*. For *age-1(mg44)* and *age-1(mg109)*, fertile adults were *age-1(mg44/mg44)* progeny of *age-1(mg44/+)* hermaphrodites. Crosses, circles and triangle shapes represent tissue-specific *age-1* or *daf-16* expression, as indicated.

Table 7
FIRE response in animals with tissue-restricted *age-1* and *gfpdaf-16* expression

Genotype	Intestinal esterase distribution (6 hour fast)			(*n*)	Rescue (Y/P/N)
	Nuclear	Intermediate [a]	Cytoplasm		
age-1(+)	100%	0%	0%	44	
age-1(mg44) m+z-	3%	41%	56%	24	
age-1(mg305)	0%	6%	94%	18	
age-1(mg109) m+z-	2%	30%	68%	59	
daf-16(mg242); age-1(m109)	70%	21%	9%	70	
Pric-19:age-1					
mg44; bvEx12	0%	0%	100%	11	N
mg44; bvIs2	0%	18%	82%	11	N
mg44; bvEx123	0%	12%	88%	26	N
mg44; bvEx121	0%	7%	93%	15	N
mg44; bvEx125	0%	60%	40%	15	P
mg44; bvEx128	0%	8%	92%	12	N
Pflp-1:age-1					
mg44;bvEx47	34%	9%	56%	32	P
Punc-4:age-1					
mg44;bvEx61	31%	31%	38%	13	P
mg305;bvEx75	20%	30%	50%	10	P
mg305;bvEx78	6%	13%	81%	16	N
Punc-25:age-1					
mg305; bvEx67	10%	10%	80%	10	N
mg305; bvEx68	0%	6%	94%	16	N
mg305; bvEx69	10%	50%	40%	10	P
mg305; bvEx70	0%	80%	20%	5	P
Pspl-1:age-1					
mg44;bvEx87	25%	38%	38%	8	P
mg44; bvEx88	25%	50%	25%	4	P
mg44; bvEx90	6%	19%	75%	16	P
Pdpy-30:daf-16					
daf-16(-); age-1(-); bvEx130	27%	73%	0%	15	P
Pric-19:daf-16					
daf-16(-); age-1(-); bvEx94	59%	41%	0%	17	N
Pric-19:daf-16 + Pgly-19:daf-16					
daf-16(-); age-1(-); bvEx113	28%	72%	0%	18	P
daf-16(-); age-1(-); bvEx114	35%	60%	4%	23	P

[a] Both nuclear and cytoplasmic esterase activity detected throughout intestine.

However, we note that the *daf-16* transgenes generated for our study provided lower levels of *daf-16* activity than previous reported (Libina et al., 2003). For example, *Pdpy-30:gfp:daf-16* only increased *daf-16(mg242)*; *age-1(mg109)* lifespan by 25%, although full rescue should have increased lifespan by 46-59% (Table 6). In addition, the previous study reported greater effects of intestinal *daf-16* activity on lifespan (Libina et al., 2003). One difference between these studies is the genetic backgound used for the rescue analysis. We examined the effect of *daf-16* expression in *daf-16 (mg242)*; *age-1(mg109)* animals, while the previous study examined the *daf-16(mu86)*; *daf-2(e1370)* background. The *daf-16(mg242)* allele is a nonsense mutation at amino acid 220 in the DAF-16 DNA binding domain that also affects both the DAF-16a and b forms (Gami and Wolkow, in preparation). The *daf-16(mu86)* allele is a large, internal deletion in the *daf-16* gene which could provide partial *daf-16* activity as a result of aberrant splicing. Residual *daf-16* activity in the *daf-16(mu86)* background could complicate interpretation of transgenic activity. Alternatively, there could be AGE-1-independent effectors of DAF-2 signaling that may be active in the *age-1(mg109)* background, but inactive in the *daf-2(e1370)* background. In this scenario, the activity of transgenic DAF-16 may be limited by residual DAF-2 signaling, through such AGE-1-independent pathways. Further analysis of *daf-16* function and regulation is needed to fully resolve these issues.

FIRE: An assay for monitoring insulin signaling at the cellular level in C. elegans

This work describes a novel assay for measuring cellular effects of insulin signaling by monitoring an intestinal cellular response to fasting. In well-fed animals, gut esterase activity was detectable throughout the intestinal cell cytoplasm, but short-term fasting caused esterase activity to redistribute to the nucleus, which we refer to as the FIRE response. In wildtype animals, the FIRE response was also observed after exposure to high temperature (35°) or to the free-radical generator, paraquat, indicating that the FIRE response may also be regulated by cellular stress (W. Iser and C. Wolkow, unpublished observations). The mechanistic basis for the FIRE response has not yet been determined. One possibility is that this response reflects fasting- and stress-induced alterations in cellular trafficking pathways.

The effects of tissue-restricted insulin signaling on stress resistance have not been investigated to date. Analysis of stress resistance has traditionally been measured by whole-organism survival using assays similar in design to lifespan assays (Larsen, 1993; Lithgow et al., 1995). Many of the genes required for stress resistance in *daf-2* mutants are also required for long lifespan (Hsu et al., 2003; Morley and Morimoto, 2004; Walker and Lithgow, 2003). It was therefore expected that stress resistance and lifespan would be linked. Interestingly, our analysis indicates that the FIRE response may be separable from other insulin outputs. For example, neuron-restricted *age-1* activity could promote wildtype development and lifespan, but did not affect the altered FIRE response of the *age-1(-)* background.

We found that the FIRE response was partially affected by *age-1* expression from some neuron-restricted promoters, such as *flp-1* and *unc-4* (Table 7). This could reflect promiscuous intestinal expression of these promoters or weak regulation of the FIRE response by the endocrine-like outputs of neuronal

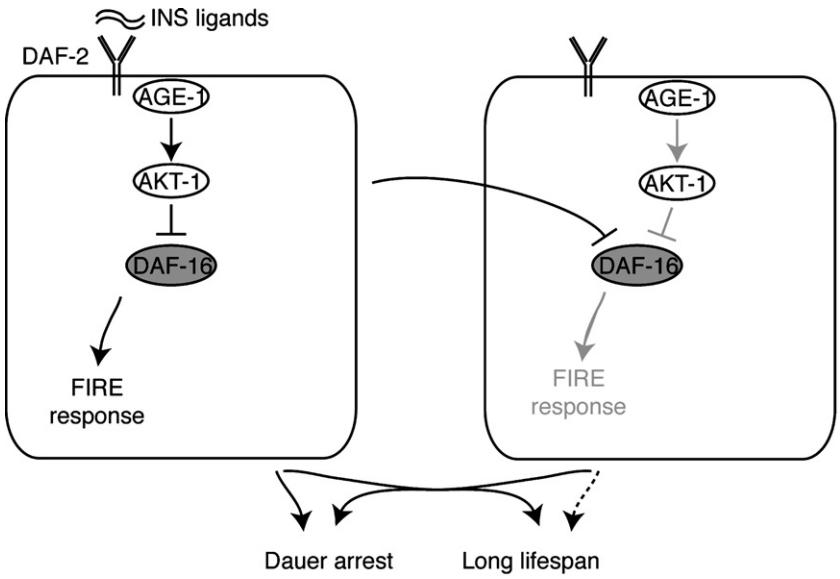

Fig. 7. A model for tissue-intrinsic and endocrine effects of insulin. In this model, insulin signaling within one cell (left) activates several *daf-16*-dependent outputs, including diffusible signals that may impinge on *daf-16* activity in distant cells where insulin signaling is inactive (right cell). Insulin regulation of the FIRE response appears to occur preferentially via cell-autonomous pathways. The diffusible outputs of insulin can affect lifespan without necessarily affecting cell-intrinsic outputs in distant cells.

insulin signaling. To address this issue, we investigated whether there was an obvious correlation between lifespan and FIRE among individual *age-1* and *daf-16* transgenic lines (Fig. 6, Table 7). First, we found that the *Pric-19:age-1* and *Pric-19:gfpdaf-16* transgenes did not strongly affect FIRE, although *Pric-19:age-1* rescued long lifespan of *age-1(-)* animals (Figs. 6A, D). This suggests that the *Pric-19* promoter is specifically expressed in the nervous system, and is consistent with the hypothesis that FIRE is an intestine-specific phenotype. Second, the strongest effects on FIRE were observed in lines with intestinal *age-1* or *daf-16* expression, as well as lines with *Punc-4:age-1* and *Pflp-1:age-1* (Figs. 6 B, C, D). This suggests that the *unc-4* and *flp-1* promoters may also be intestinally expressed. We note that the *Punc-4:age-1* and *Pflp-1:age-1* lines with the strongest effect on FIRE also significantly shortened lifespan, which was not observed in *Pspl-1:age-1* lines. Although the basis for shortened lifespan in *Punc-4:age-1* and *Pflp-1:age-1* lines is not yet known, it appears to correlate with strong effects on FIRE.

Lifespan may result from incorporation of cell-autonomous and endocrine-like outputs of insulin signaling

Our transgenic experiments support the existence of a non-cell autonomous, endocrine-like pathway through which insulin promotes reproductive development and wildtype lifespan. Furthermore, our studies show that this endocrine-like effect can arise from insulin signaling in a number of different tissues and has a graded effect on lifespan and development. However, we found that tissue-restricted *daf-16* expression did not affect lifespan as strongly as *daf-16* expression in multiple tissues. This may reflect a requirement for *daf-16* activity in most or all tissues in order for insulin signaling decrements to extend lifespan. One model to accommodate this data may be that insulin signaling regulates the production of diffusible factors that impinge on DAF-16 activity throughout the body. Consistent with this idea, DAF-16 nuclear localization was affected non-cell autonomously by DAF-18/PTEN activity, suggesting that insulin signaling can affect DAF-16 activity in distant cells (Masse et al., 2005).

One pathway for the non-cell autonomous regulation of DAF-16 could be feed-back regulation of insulin (*ins*) production or secretion. Insulin signaling has been shown to regulate expression of at least one insulin-like ligand, *ins-7* (Murphy et al., 2003). However, such feedback regulation of insulin ligands cannot explain how neuron-restricted insulin signaling could rescue lifespan when non-neuronal tissues lacked a functional insulin signaling pathway. Therefore, we favor an alternative model in which DAF-2 signaling can regulate DAF-16 in the same cells, via AKT phosphorylation, and in distant cells, through a diffusible signal. Indeed, several pathways can regulate DAF-16 independently of DAF-2, including Jun N-terminal kinase JNK-1, beta-catenin BAR-1 and the heat-shock transcription factor HSF-1 (Essers et al., 2005; Hsu et al., 2003; Oh et al., 2005). Interactions between these proteins and insulin signaling could provide pathways for coordinating DAF-16 activity throughout the body in response to insulin signaling in a subset of cells.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ydbio.2006.04.467.

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